

Deletion of Marek's Disease Virus Large Subunit of Ribonucleotide Reductase Impairs Virus Growth *In Vitro* and *In Vivo*

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SUMMARY. Marek's disease virus (MDV), a highly cell-associated lymphotropic alphaherpesvirus, is the causative agent of a neoplastic disease in domestic chickens called Marek's disease (MD). In the unique long (UL) region of the MDV genome, open reading frames UL39 and UL40 encode the large and small subunits of the ribonucleotide reductase (RR) enzyme, named RR1 and RR2, respectively. MDV RR is distinguishable from that present in chicken and duck cells by monoclonal antibody T81. Using recombinant DNA technology we have generated a mutant MDV ($Md5\Delta RR1$) in which RR1 was deleted. PCR amplification of the RR gene in $Md5\Delta RR1$ -infected duck embryo fibroblasts (DEF) confirmed the deletion of the 2.4 kb RR1 gene with a resultant amplicon of a 640-bp fragment. Restriction enzyme digests with *Sall* confirmed a UL39 deletion and the absence of gross rearrangement. The biologic characteristics of $Md5\Delta RR1$ virus were studied *in vitro* and *in vivo*. The $Md5\Delta RR1$ replicated in DEF, but significantly slower than parental $Md5$ -BAC, suggesting that RR is important but not essential for replication in fibroblasts. *In vivo* studies, however, showed that the RR1 deletion virus was impaired for its ability to replicate in chickens. Inoculation of specific-pathogen-free (SPF) chickens with $Md5\Delta RR1$ showed the mutant virus is nonpathogenic and does not induce MD in birds. A revertant virus, $Md5\Delta RR1/R$, was generated with the restored phenotype of the parental $Md5$ -BAC *in vivo*, indicating that RR is essential for replication of the virus in chickens. Protection studies in SPF chickens indicated that the $Md5\Delta RR1$ virus is not a candidate vaccine against MD.

RESUMEN. La delección de la subunidad mayor de la ribonucleótido reductasa (RR) del virus de la enfermedad de Marek afecta el crecimiento del virus *in vitro* e *in vivo*.

El virus de la enfermedad de Marek (MDV), un alfaherpesvirus linfotrópico altamente asociado a células, es el agente causal de una enfermedad neoplásica en pollos domésticos llamada enfermedad de Marek (MD). En la región única larga (UL) del genoma del virus de Marek, los marcos de lectura continuos UL39 y UL40 codifican las subunidades mayor y menor de la enzima ribonucleótido reductasa (RR), denominadas RR1 y RR2, respectivamente. La enzima ribonucleótido reductasa del virus de Marek es distinguible de la presente a partir en las células de pollo y pato mediante el anticuerpo monoclonal T81. Con el uso de tecnología de ADN recombinante se ha generado un virus de Marek ($Md5\Delta RR1$) en el cual la subunidad RR1 ha sido eliminada. La amplificación mediante PCR del gene RR en fibroblastos de embrión de pato (DEF) infectados por el virus $Md5\Delta RR1$, confirmó la delección de la subunidad RR1 de 2.4 kb con un amplicón resultante de un fragmento de 640 pb. La digestión con la enzima de restricción *Sall* confirmó una delección del UL39 y una ausencia de reordenamiento. Las características biológicas del virus $Md5\Delta RR1$ se estudiaron *in vitro* e *in vivo*. El virus $Md5\Delta RR1$ se replicó en fibroblastos de embrión de pato, pero su replicación fue significativamente más lenta que la del virus progenitor $Md5$ -BAC, lo que sugiere que la enzima ribonucleótido reductasa es importante pero no esencial para la replicación en los fibroblastos. Sin embargo en estudios *in vivo*, se demostró que el virus con delección de la subunidad RR1 resultó afectado en su capacidad para replicarse en pollos. La inoculación de pollos libres de patógenos específicos (SPF) con el virus $Md5\Delta RR1$ mostraron que el virus mutante no es patógeno y no induce la enfermedad de Marek en las aves. Un virus revertiente, $Md5\Delta RR1 / R$, se generó con el fenotipo restaurado del virus progenitor $Md5$ -BAC *in vivo*, lo que indica que la ribonucleótido reductasa es esencial para la replicación del virus en los pollos. Estudios de protección en pollos libres de patógenos específicos indicaron que el virus $Md5\Delta RR1$ no es un candidato para elaborar una vacuna contra la enfermedad de Marek.

Key words: Marek's disease virus, bacterial artificial chromosome, ribonucleotide reductase, UL39, $Md5$ strain, pathogenesis

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; BAC = bacterial artificial chromosome; CEF = chicken embryo fibroblasts; DEF = duck embryo fibroblasts; HSV = herpes simplex virus; IFA = immunofluorescence assay; Kan^R = kanamycin resistance gene; MD = Marek's disease; MDV = Marek's disease virus; MDV-1 = MDV serotype 1; PBS = phosphate-buffered saline; PMLB = peripheral mononuclear blood leukocytes; PFU = plaque-forming units; PRV = pseudorabies virus; RR = ribonucleotide reductase; RR1 = large subunit of RR; RR2 = small subunit of RR; UL = unique long region of MDV genome; vv+ = very virulent plus

Marek's disease (MD), a lymphoproliferative disease of domestic chickens (6,19), is caused by *Gallid herpesvirus 2* (GaHV-2) or MD virus (MDV serotype 1 or MDV-1), a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Mardivirus* (9). Two other members in the genus *Mardivirus* are *Gallid herpesvirus 3* (previously known as MDV serotype 2 [MDV-2]) and *Meleagrid herpesvirus 1* (previously known

as MDV serotype 3 or HVT), which are commonly used as vaccines to control MD. The molecular structure and genomic organization of MDV is very similar to herpes simplex virus (HSV), hence its classification within the *Alphaherpesviridae* family (1,14,17,28). MDV includes three serotypes that have major differences not only in the genome but also in biological features (4). MDV-1 includes all the oncogenic strains and their attenuated forms. The oncogenic strains cause an acute T-cell lymphomas in chickens; serotypes 2 and 3 are nononcogenic viruses isolated from chickens (25) and turkeys, respectively (31).

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Table 1. List of primers used for the manipulation of the Md5-BAC.

Primers	Sequences ^A	Use
RR-kana ^R -a	5'- GCGCATTGACAAATTATACGGTCATCCACTC- TGGCACTAAAAATCTGCTAGGATGACGACGATAA- <i>GTAGGG-3'</i>	Amplification of Kan ^R cassette gene with flanking RR sequences
RR-kana ^R -b	<i>5'-TTTCTGTGCGTTAGAAGAACATGATGATGGTTAC-</i> TGGCACGTGCAGGATCAGCAGATTAGTC- GCAGAGTGGATGACCGTATAATTGTCGAATGC- GC CAACCAATTAAACCAATTCTGATTAG-3'	
RR F	5'-CCGCGATCGTAGGTTGGGTATTG-3'	Amplification for RR with MDV flanking sequences
RR R	5'-ATGCGCAGTGATTGGTGGTAGGAA-3'	

^AFor primers RR-kana^R-a and RR-kana^R-b, the italic sequences indicate those from pEPkan-S used to amplify the Kan^R gene cassette, and boldface sequences indicate the MDV sequence flanking the RR gene.

MD has been successfully controlled by vaccination since 1969. The use of MD vaccines has been accompanied with the increase in virulence of MDV (29). During the last 40 years, MDV has evolved from virulent (v) to very virulent (vv) and very virulent plus (vv+) pathotypes (30). The continued evolution of MDV toward greater virulence has prompted concern that the current vaccines will ultimately lose efficacy in controlling MD. This situation has led several investigators to develop more efficacious vaccines, but it has been a difficult challenge (30). In an effort to identify genes that are common in the three serotypes of MDV, genes that could be deleted from the MDV genome and thus lead to generation of new vaccine candidates, we identified a clone in the lambda gt11 library of MDV using a serotype-common monoclonal antibody T81. Sequence analysis of this clone identified the gene as UL39, which encodes an enzyme called ribonucleotide reductase (RR).

RR exists in all eukaryotic and prokaryotic organisms and is an essential enzyme for the conversion of ribonucleotides to deoxyribonucleotides (7,15). RR expressed by herpesviruses exists as a complex of two subunits, the large (RR1) and small (RR2). Deletion of either of the RR subunits eliminates the enzyme activity (11,23). In herpesviruses the RR genes are not essential for virus growth in tissue culture and, thus, are good candidates for deletion mutants (5,12). In pseudorabies virus (PRV), a mutant deficient in RR was shown to be avirulent for pigs and capable of partially protecting pigs against the disease (10). The gene coding for MDV RR is located in the unique long (UL) region of the genome. The large subunit is encoded by UL39 (RR1) and is predicted to comprise 860 amino acids while the small subunit encoded by UL40 (RR2) is predicted to be 343 amino acids long. To our knowledge, there are no published data as to the function of the RR complex in MDV. Therefore, we set out to characterize the role of RR in MDV pathogenesis.

In recent years, manipulation of the large herpesvirus genomes, including that of MDV, has been facilitated by using bacterial artificial chromosome (BAC) (3,21,22,26) or cosmid vectors (18,24). BAC cloning of the MDV genome and mutagenesis has become an important technology in recent years for understanding the functions or biologic characteristics of MDV genes. Here we report the construction of RR deletion and revertant mutants by red-mediated recombination (27) of Md5-BAC virus and the characterization of both mutants *in vitro* and *in vivo* for their roles in the pathogenesis of MDV.

MATERIALS AND METHODS

Cells and viruses. Chick embryo fibroblast (CEF) and duck embryo fibroblast (DEF) cultures were grown at 37°C in Liebowitz-McCoy (1:1) medium supplemented with 4% calf serum. Confluent cultures were infected with cell-associated MDV at a multiplicity of infection of

approximately 0.3 and maintained in the same media with 1% calf serum. Md5BAC was kindly supplied to us by Dr. Hans Cheng from the Avian Disease and Oncology Laboratory (ADOL; U. S. Department of Agriculture), East Lansing, MI.

Chickens. Chickens used in this study were from the 151₅ × 7₁ hybrid chicks (2) free of maternal antibodies to all three serotypes of MDV. The breeder chickens were maintained at ADOL and were free of antibodies to avian leukosis virus, reticuloendotheliosis virus, and various other poultry pathogens.

Construction of RR deletion and revertant viruses from Md5-BAC. For deletion of the UL39 gene of Md5-BAC, a two-step red-mediated recombination procedure was followed (27). During the first red recombination step in *Escherichia coli*, the UL39 gene was replaced with the kanamycin resistance (Kan^R) gene amplified using RR-kanaR-a and RR-kanaR-b primers (Table 1) and plasmid pEPKan-S as a template Md5-BAC-KanR. A second red recombination step was carried out by addition of arabinose to the growth media, resulting in induction of *I-Sce* and cleavage of Md5-BAC-Kan^R, deleting the Kan^R gene, followed by recombination of the BAC sequences to generate Md5ΔRR1. Furthermore, a revertant clone (Md5ΔRR1/R) was generated in which the parental template sequence was restored in the mutant Md5ΔRR1.

Growth characteristics of recombinant virus in cell culture. Growth characteristics of Md5-BAC, Md5ΔRR1, and Md5ΔRR1/R viruses were studied as previously described (24). Briefly, approximately 100 plaque-forming units (PFU) of the different viruses were inoculated onto DEF cells seeded on 60-mm plates. On days 1, 2, 3, and 5 postinoculation, the infected cells were trypsinized, serial dilutions were inoculated onto DEF cells seeded on 35-mm plates, and plaques in the different dilutions were counted 7 days later.

Reactivation assay. Buffy-coats were obtained from heparanized blood by centrifugation at 500 × g for 5 min. Lymphocytes were then counted and diluted to 10⁶ cells/ml. For each chicken sample, duplicated 35-mm plates of freshly seeded DEF monolayers were inoculated with both 10⁵ and 10⁶ lymphocytes, and viral plaques were counted 7 days postinoculation.

Immunofluorescence assay (IFA). A method described previously was followed with modifications (16). Briefly, transfected cells were washed with phosphate-buffered saline (PBS) and fixed with methanol:acetone solution (6:4) at 37°C for 10 min. After removing fixing solution, the cells were air-dried and then incubated with MAbs H19 or T81 (1:500) for 1 hr at 37°C. Following three washes with PBS, cells were incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-labeled secondary antibodies (KPL, Gaithersburg, MD) for 1 hr. Cells were then washed 3 times with PBS and examined under a fluorescence microscope.

Pathogenesis and protection experiments. Chickens used in the study were MD-susceptible maternal-antibody-negative F1 progeny (151₅ × 7₁) of the ADOL (USDA, East Lansing, MI) line 151₅ males and line 7₁ females. These chickens were wing-banded at hatch, randomly sorted into five experimental groups of 17–21 chickens each, and held in modified Horsfall-Bauer isolators for the duration of the experiment. One of the chicken lots remained as the uninoculated

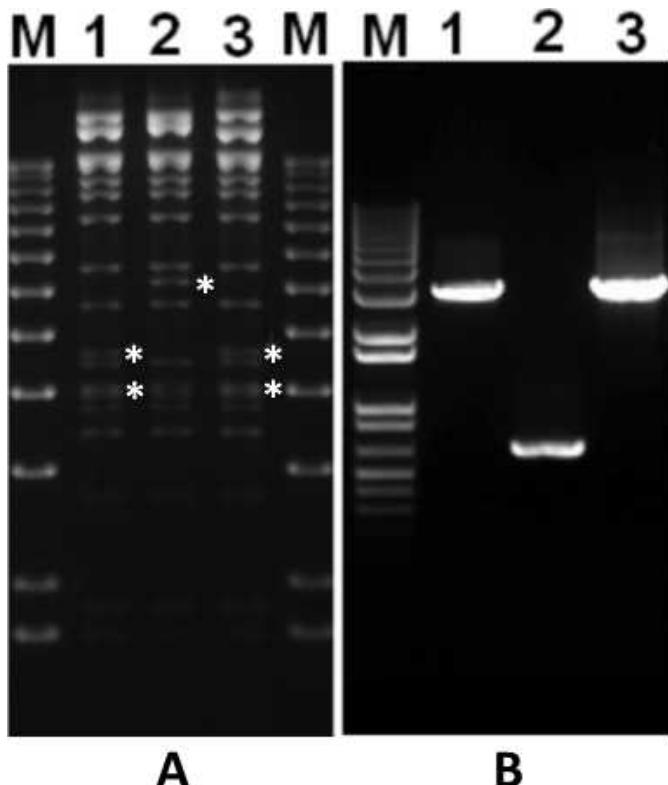


Fig. 1. Genomic structure of RR deletion mutant. (A) Restriction enzyme digestion of BAC DNA: Md5-BAC (lane 1), Md5 Δ RR1 (lane 2), and Md5 Δ RR1/R (lane 3). BAC DNA was digested with SallI, separated by gel electrophoresis in 1% agarose gel, and stained with ethidium bromide. Asterisks indicate expected bands of 3.9 and 4.5 kb in Md5-BAC and Md5 Δ RR1/R and of 6 kb in Md5 Δ RR1 (due to deletion of the RR1 gene and loss of Sall site within). (B) PCR analysis to confirm RR1 was deleted in Md5 Δ RR1 (lane 2; 0.64-kb amplicon) and present in Md5-BAC and Md5 Δ RR1/R (lanes 1 and 3, respectively; 3.1-kb amplicon). Primers used were those listed in Table 1. M = 1 kb DNA standard (Life Technologies, Carlsbad, CA).

control group. The rest of three lots were inoculated intra-abdominally with 2000 PFU of either Md5-BAC, or Md5 Δ RR1, or Md5 Δ RR1/R at 1 day of age. An additional lot was vaccinated with vv+686 MDV. All birds that died during the trial or were killed at the end of the experiment (8 wk after inoculation) were necropsied and evaluated for gross lesions. To study the effect of the RR deletion on early cytolytic infection, three chickens each from the infected groups and the uninoculated control group were euthanized 6 days postinoculation and lymphoid organs (thymus, bursa of Fabricius, and spleen) collected and examined for viral antigen expression (pp38 and RR) by immunohistochemistry. To study virus reactivation from latency, two chickens from the control group and 5 chickens each from the infected groups were bled on day 21 postinoculation for a reactivation assay. To study the protection efficacy of Md5 Δ RR1, a vv+MDV strain, 686, was used as a challenge virus 5 days postvaccination.

RESULTS

Deletion of RR from rMd5-BAC. UL39, the gene encoding RR, was deleted from Md5-BAC (20) using the two-step red-mediated recombination method described earlier (27) to generate Md5 Δ RR1. Viruses were reconstituted by transfection of Md5 Δ RR1 DNA and Md5-BAC into CEF. To investigate whether the two-step red-mediated recombination procedure introduced any unwanted major

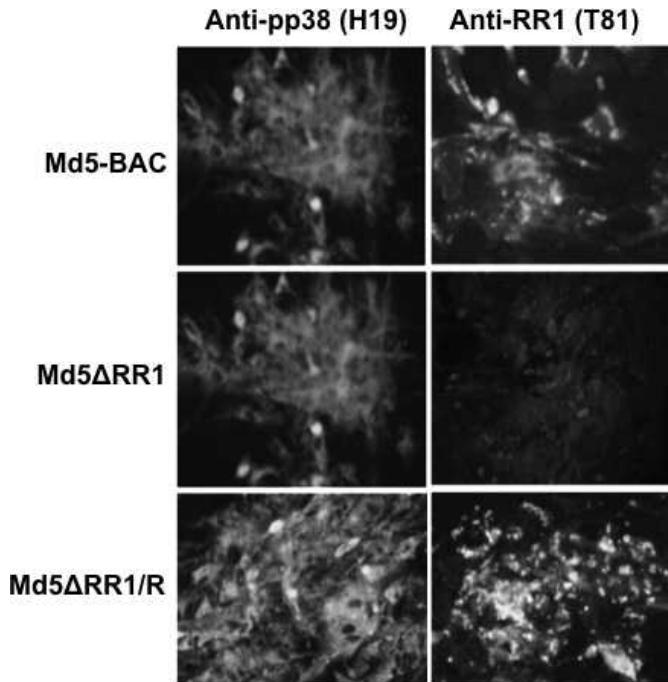


Fig. 2. IFA of MDV plaques after transfection of CEFs with Md5-BAC, Md5 Δ RR1, or Md5 Δ RR1/R DNA. At 5 days postinfection, cells were fixed and subjected to IFA using anti-RR and anti-pp38 monoclonal antibodies (T81 and H19, respectively). There were no differences between the three viruses when monoclonal antibody H19 was used. No expression of RR was detected with monoclonal antibody T81 in Md5 Δ RR1-infected cells but was detected in Md5-BAC- and Md5 Δ RR1/R-infected cells.

rearrangements or deletions, DNA from these viruses was digested with SallI and the restriction digestion pattern examined in a 1% agarose gel stained with ethidium bromide. As seen in Figure 1A, SallI digestion of parental and revertant DNA (lines 1 and 3, respectively) produced bands of 3.9 and 4.5 kb (indicated with asterisks). Deletion of RR1 (lane 2) resulted in the elimination of a Sall site, and the disappearance of the 3.9 and 4.5 kb bands, as well as generation of a new band of 6 kb (indicated with an asterisk). In addition, to confirm that the UL39 coding region had been deleted, PCR amplification was carried out across the UL39 gene. As shown in Figure 1B, parental Md5-BAC (lane 1) and revertant Md5 Δ RR1/R DNA (lane 3) produced a 3.1-kb amplicon while Md5 Δ RR1 DNA (lane 2) produced a 0.64-kb product.

To confirm further the deletion of RR1 gene, virus-infected cells were examined by IFA with anti-pp38 monoclonal antibody H19 and anti-RR1 monoclonal antibody T81. As expected, Md5-BAC and Md5 Δ RR1/R expressed both pp38 and RR1 while Md5 Δ RR1 expressed pp38 but not RR1 (Fig. 2).

In vitro replication of rMd5 Δ RR1. To determine whether the deletion of the RR1 gene had any effect on virus replication *in vitro*, the growth rate of rMd5 Δ RR1 virus was compared with that of Md5-BAC and the revertant virus. As shown in Figure 3, Md5 Δ RR1 exhibited decreased replication in DEF, while the growth characteristics of Md5-BAC and Md5 Δ RR1/R were similar at all time points tested.

Reactivation assay. Groups of 1-day-old chickens were inoculated with Md5-BAC, Md5 Δ RR1, and Md5 Δ RR1/R. Another group of chickens was inoculated with Md5 Δ RR1 at 1 day and challenged with vv+686 at 5 days after inoculation. Five chickens from each group were bled at 21 days postchallenge and cell-associated

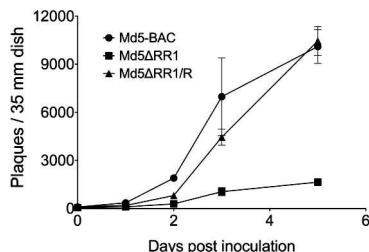


Fig. 3. Growth properties of recombinant viruses in cell culture. DEF cells were infected with approximately 100 PFU of Md5-BAC, Md5ΔRR1, and Md5ΔRR1/R. Virus titers were determined at the indicated times postinfection in fresh DEF cells. Each time point represents the mean of two independent experiments. Bars represent standard error.

MDV viremia was determined. As shown in Figure 4, there was no virus isolated from the RR1 deleted virus (Md5ΔRR1), whereas the Md5-BAC, 686, and revertant viruses Md5ΔRR1/R had about 90 PFU/1 \times 10⁶ buffy-coat cells. The Md5ΔRR1 group challenged with vv+686 also had about 50 PFU/1 \times 10⁶ buffy-coat cells.

RR deletion mutant virus Md5ΔRR1 is attenuated. The pathogenesis of the mutant virus Md5ΔRR1 and the revertant virus Md5ΔRR1/R were compared with parental virus Md5-BAC in MDV maternal-antibody-negative chickens (Table 2). Chickens inoculated with Md5ΔRR1, Md5ΔRR1/R, and Md5-BAC were observed daily for a period of 8 wk. During the experimental period, no gross lesions or mortality were observed in the group inoculated with Md5ΔRR1. High mortality was observed in the groups inoculated with Md5-BAC or Md5ΔRR1/R. Based on the results of the mortality and gross lesions, deletion of the RR1 gene had significantly attenuated the virulence of Md5-BAC.

Recombinant virus Md5ΔRR1 did not have potential as a vaccine against MD. Tests to determine the protective efficacy of Md5ΔRR1 were carried out on MDV maternal-antibody-negative chickens. The incidence of MD in chickens vaccinated with Md5ΔRR1 with vv+686 was 68% compared to 100% in the nonvaccinated challenge group. Both Md5ΔRR1 and Md5ΔRR1/R induced 88% MD and vv+686 induced 100% MD. The protection afforded by the RR1 deletion virus against vv+686 challenge was about 32%.

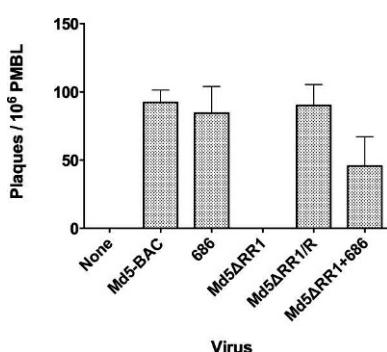


Fig. 4. Reactivation assay. Five chickens from each virus-inoculated group were bled at 21 days postchallenge and cell-associated MDV viremia was determined and expressed as PFU/10⁶ PMBL. Bars represent standard error.

Table 2. Evaluation of Md5ΔRR1 pathogenicity and protection efficacy in chickens.

Viruses ^A	686 challenge ^B	Mortality	Total MD mortality	% MD ^C
Md5-BAC	—	15/17	15/17	88a,b
Md5ΔRR1	—	0/17	0/17	0c
Md5ΔRR1	+	11/19	13/19	68b
Md5ΔRR1/R	—	11/12	15/17	88a,b
None	+	17/17	17/17	100a
None	—	0/14	0/14	0

^AAll chickens were inoculated with 1,500 PFU of the indicated virus.

^BChickens were challenged with 500 PFU of 686 vv+ MDV virus.

^CDifferent lowercase letters differ significantly based on chi-square analysis ($P < 0.05$).

DISCUSSION

RR is an essential enzyme for nucleotide metabolism. It converts ribonucleotides to deoxyribonucleotides and, thus, increases the deoxynucleotide triphosphate (dNTP) pool for viral DNA synthesis in infected cells (7). All herpesviruses encode their own RR and the enzyme may be essential for viral pathogenesis (13). In HSV, an RR mutant showed reduced virulence, altered latency, and reactivation in the mouse model (5). Similarly, RR mutants of pseudorabies virus were avirulent for pigs and provided partial protective immunity against virulent challenges (10). In this study we sought to investigate the role of RR in MDV pathogenesis.

In order to study the biologic function of MDV RR, using BAC technology we generated a mutant virus lacking the large subunit of RR, Md5ΔRR1. As in HSV-1 and *Varicella zoster virus* (VZV) RR deletion mutants (5,13), *in vitro* growth kinetics of Md5ΔRR1 showed that deletion of the RR gene affected the rate of virus growth, suggesting that RR is required but not essential for effective replication in cell culture. The generation of a revertant virus confirmed that all the observed biologic differences between the deletion mutant and parental Md5-BAC were the result of the deleted RR gene and not from other unexpected mutations in the viral genome.

During the early stage of lytic infection (4 to 7 days after infection), B-cells and some T-cells support a cell-associated productive MDV infection in chickens, and the lymphoid tissues become the major site of virus replication. To examine the role of RR in early cytotolytic infection, Md5-BAC and Md5ΔRR1 viruses were inoculated into MDV Ab- chickens at 1 day of age. Our results show that at 6 days after inoculation there was significant expression of viral antigens in the lymphoid organs of Md5-BAC-inoculated birds but not in Md5ΔRR1-inoculated chickens (data not shown), indicating that RR is essential for establishment of early cytotolytic infection in lymphocytes.

It is generally assumed that a successful cytotolytic infection in B- and T-cells is prelude to latent-infection and transformation of T-cells. However, exceptions to this assumption are the pp38 (24) or the viral interleukin 8 (vIL-8) (8) deletion rMd5 viruses, both of which were impaired for early cytotolytic infection but exhibited latent infection leading to transformation. However, virus reactivation studies of Md5ΔRR1 showed no virus recovery from the peripheral mononuclear blood leukocytes (PMBL) of infected chickens, while parental and RR revertant viruses had significant viral titers at 21 days postinoculation. In addition, none of the Md5ΔRR1-infected chickens developed MD while 88% of chickens infected with Md5-BAC or RR revertant virus developed MD. All together, these data suggest that in addition to affecting early cytotytic

infection in lymphoid organs, RR may also affect MDV latency and subsequent tumor induction.

Because Md5ΔRR1 was attenuated, we evaluated whether this nonpathogenic virus could be used as vaccine against vv+MDV challenge. Vaccination of chickens with Md5ΔRR1 conferred a partial protection (32%) upon challenge with vv+686 (Table 2); this may be attributable to limited replication of Md5ΔRR1 in nonlymphoid cells in chickens. These data are in agreement with results obtained for the RR deletion mutant PRV virus in pigs, confirming that RR is not a good target for development of herpesvirus vaccines (10).

In summary, RR is essential for MDV early cytopathic infection in B- and T-cells, as well as for latency, reactivation, or both. In addition, Md5ΔRR1 virus was so highly attenuated in chickens that it provided only limited protection against challenge with a vv+ virus and, therefore, cannot be used as a vaccine.

REFERENCES

- Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, D. L. Rock, and G. F. Kutish. The genome of turkey herpesvirus. *J. Virol.* 75:971–978. 2001.
- Bacon, L. D., H. D. Hunt, and H. H. Cheng. A review of the development of chicken lines to resolve genes determining resistance to diseases. *Poult. Sci.* 79:1082–1093. 2000.
- Baigent, S. J., L. J. Petherbridge, L. P. Smith, Y. Zhao, P. M. Chesters, and V. K. Nair. Herpesvirus of turkey reconstituted from bacterial artificial chromosome clones induces protection against Marek's disease. *J. Gen. Virol.* 87:769–776. 2006.
- Bulow, V. V., and P. M. Biggs. Differentiation between strains of Marek's disease virus and turkey herpesvirus by immunofluorescence assays. *Avian Pathol.* 4:133–146. 1975.
- Cameron, J. M., I. McDougall, H. S. Marsden, V. G. Preston, D. M. Ryan, and J. H. Subak-Sharpe. Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. *J. Gen. Virol.* 69(Pt. 10):2607–2612. 1988.
- Churchill, A. E., and P. M. Biggs. Agent of Marek's disease in tissue culture. *Nature* 215:528–530. 1967.
- Conner, J., A. Cross, J. Murray, and H. Marsden. Identification of structural domains within the large subunit of herpes simplex virus ribonucleotide reductase. *J. Gen. Virol.* 75(Pt. 12):3327–3335. 1994.
- Cui, X., L. F. Lee, W. M. Reed, H. J. Kung, and S. M. Reddy. Marek's disease virus-encoded vIL-8 gene is involved in early cytopathic infection but dispensable for establishment of latency. *J. Virol.* 78:4753–4760. 2004.
- Davison, A. J., R. Eberle, B. Ehlers, G. S. Hayward, D. J. McGeoch, A. C. Minson, P. E. Pellett, B. Roizman, M. J. Studdert, and E. Thiry. The order Herpesvirales. *Arch. Virol.* 2008.
- de Wind, N., A. Berns, A. Gielenkens, and T. Kimman. Ribonucleotide reductase-deficient mutants of pseudorabies virus are avirulent for pigs and induce partial protective immunity. *J. Gen. Virol.* 74(Pt. 3):351–359. 1993.
- Goldstein, D. J., and S. K. Weller. Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. *Virology* 166:41–51. 1988.
- Goldstein, D. J., and S. K. Weller. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. *J. Virol.* 62:196–205. 1988.
- Heineman, T. C., and J. I. Cohen. Deletion of the varicella-zoster virus large subunit of ribonucleotide reductase impairs growth of virus in vitro. *J. Virol.* 68:3317–3323. 1994.
- Izumiya, Y., H. K. Jang, M. Ono, and T. Mikami. A complete genomic DNA sequence of Marek's disease virus type 2, strain HPRS24. *Curr. Top. Microbiol. Immunol.* 255:191–221. 2001.
- Jordan, A., and P. Reichard. Ribonucleotide reductases. *Annu. Rev. Biochem.* 67:71–98. 1998.
- Lee, L. F., X. Liu, and R. L. Witter. Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. *J. Immunol.* 130:1003–1006. 1983.
- Lee, L. F., P. Wu, D. Ren, J. Kamil, H. J. Kung, and R. L. Witter. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. *Proc. Natl. Acad. Sci. U. S. A.* 97:6091–6096. 2000.
- Lupiani, B., L. F. Lee, X. Cui, I. Gimeno, A. Anderson, R. W. Morgan, R. F. Silva, R. L. Witter, H. J. Kung, and S. M. Reddy. Marek's disease virus-encoded Meq gene is involved in transformation of lymphocytes but is dispensable for replication. *Proc. Natl. Acad. Sci. U. S. A.* 101:11815–11820. 2004.
- Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester. Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. *Proc. Soc. Exp. Biol. Med.* 127:177–182. 1968.
- Niikura, M., T. Kim, R. F. Silva, J. Dodgson, and H. H. Cheng. Virulent Marek's disease virus generated from infectious bacterial artificial chromosome clones with complete DNA sequence and the implication of viral genetic homogeneity in pathogenesis. *J. Gen. Virol.* 92:598–607. 2011.
- Petherbridge, L., A. C. Brown, S. J. Baigent, K. Howes, M. A. Sacco, N. Osterrieder, and V. K. Nair. Oncogenicity of virulent Marek's disease virus cloned as bacterial artificial chromosomes. *J. Virol.* 78:13376–13380. 2004.
- Petherbridge, L., H. Xu, Y. Zhao, L. P. Smith, J. Simpson, S. Baigent, and V. Nair. Cloning of Gallid herpesvirus 3 (Marek's disease virus serotype-2) genome as infectious bacterial artificial chromosomes for analysis of viral gene functions. *J. Virol. Methods* 158:11–17. 2009.
- Preston, V. G., A. J. Darling, and I. M. McDougall. The herpes simplex virus type 1 temperature-sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* 167:458–467. 1988.
- Reddy, S. M., B. Lupiani, I. M. Gimeno, R. F. Silva, L. F. Lee, and R. L. Witter. Rescue of a pathogenic Marek's disease virus with overlapping cosmid DNAs: use of a pp38 mutant to validate the technology for the study of gene function. *Proc. Natl. Acad. Sci. U. S. A.* 99:7054–7059. 2002.
- Schat, K. A., and B. W. Calnek. Characterization of an apparently nononcogenic Marek's disease virus. *J. Natl. Cancer Inst.* 60:1075–1082. 1978.
- Schumacher, D., B. K. Tischer, W. Fuchs, and N. Osterrieder. Reconstitution of Marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant. *J. Virol.* 74:11088–11098. 2000.
- Tischer, B. K., J. von Einem, B. Kaufer, and N. Osterrieder. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. *Biotechniques* 40:191–197. 2006.
- Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, D. L. Rock, and G. F. Kutish. The genome of a very virulent Marek's disease virus. *J. Virol.* 74:7980–7988. 2000.
- Witter, R. L. Increased virulence of Marek's disease virus field isolates. *Avian Dis.* 41:149–163. 1997.
- Witter, R. L., B. W. Calnek, C. Buscaglia, I. M. Gimeno, and K. A. Schat. Classification of Marek's disease viruses according to pathotype: philosophy and methodology. *Avian Pathol.* 34:75–90. 2005.
- Witter, R. L., K. Nazerian, H. G. Purchase, and G. H. Burgoyne. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. *Am. J. Vet. Res.* 31:525–538. 1970.

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